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Sourdough-type propagation of faba bean flour: dynamics of microbial consortia and biochemical implications

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Abstract

The microbial ecology of faba bean sourdoughs obtained from an Italian (Ita) and a Finnish (Fi) cultivar, belonging respectively to *Vicia faba major* and *V. faba minor* groups, was described by 16S rRNA gene pyrosequencing and culture-dependent analysis. The sourdoughs were propagated with traditional backslopping procedure throughout 14 days. Higher microbial diversity was found in the sourdough deriving from *V. faba minor* (Fi), still containing residual hulls after the milling procedure. After 2 days of propagation, the microbial profile of Ita sourdough was characterized by the dominance of the genera *Pediococcus*, *Leuconostoc* and *Weissella*, while the genera *Lactococcus*, *Lactobacillus* and *Escherichia*, as well as *Enterobacteriaceae* were present in Fi sourdoughs. Yeasts were in very low cell density until the second backslopping and were not anymore found after this time by plate count or pyrosequencing analysis. Among the lactic acid bacteria isolates, *Pediococcus pentosaceus*, *Leuconostoc mesenteroides* and *Weissella koreensis* had the highest frequency of occurrence in both the sourdough. *Lactobacillus sakei* was the only lactobacillus isolated from the first to the last propagation day in Fi sourdough. According to microbiological and acidification properties, the maturity of the sourdoughs was reached after 5 days. The presence of hulls and the different microbial composition reflected on biochemical characteristics of Fi sourdoughs, including acidification and phenolic compounds. Moreover, proteolysis in Fi sourdough was more intense compared to Ita. The microbial dynamic of the faba bean sourdoughs showed some differences with the most studied cereal sourdoughs.

Keywords: faba bean, sourdough, lactic acid bacteria

Abbreviations: DY, Dough Yield; Fi, Finnish; Ita, Italian; ITS, Internal Transcribed Spacer; OTUs, operational taxonomic units; RAPD, Random Amplified Polymorphic DNA; RFOs, Raffinose Family Oligosaccharides; TFAA, Total Free Amino Acids; TTA, Total Titratable Acidity; WSE, Water/salt-soluble extracts.

1. Introduction

The regular intake of plant-based foods is recommended to partially replace animal proteins in the diet. Beneficial repercussions on human health, contribution for sustaining the worldwide demand for proteins, and mitigation of the environmental burden of agricultural practices are some of the key reasons for this recommendation (De Boer & Aiking**,** 2011; Multari et al., 2015). Traditionally, legumes are considered valuable substitutes of meat in the human diet because of their high content of protein, low cost and easy availability. Faba bean (*Vicia faba L.*) is a multipurpose grain legume, employed worldwide for food and feed (Jezierny et al., 2010). The high content of protein and fiber, and the presence of many bioactive compounds indicate the potential role of faba bean in maintaining human health and disease prevention (Crépon et al., 2010; Fruhbeck et al., 1999). Indeed, faba bean has been subjected to several studies in the last decade. In particular, efforts were focused on decreasing anti-nutritional compounds which reduce the seed digestibility and lead to some pathologic conditions (Gupta, 1987). Among these, raffinose family oligosaccharides (RFOs), tannins, phytic acid and the pyrimidine glycosides, vicine and convicine were the most studied, and their content was reduced through technological and agronomic practices (Multari et al., 2015). Food processing such as air classification, soaking, cooking, germination and fermentation (Coda et al., 2015; Luo et al., 2009; Sharma & Sehgal, 1992) have addressed efficiently the reduction of these anti-nutritional compounds. For instance, fermentation with lactic acid bacteria has been one of the preferred strategy to decrease the content of RFOs in soy and other legumes, particularly in reference to their α-galactosidase activity (Duszkiewicz-Reinhard et al., 1994; Savoy de Giori et al., 2010), leading to improved digestibility. Generally, fermentation of legumes (Granito et al., 2002) and faba bean, is known to enhance the overall nutritional quality, without severe repercussion on its sensory properties (Coda et al., 2015), and can be considered an efficient way to increase its use in the food industry.

Despite this renewed interest, other efforts should be done to promote the use of faba bean for products of optimal nutritional value and consumer acceptability. Traditionally, spontaneous

88 fermentation of legumes is used in many countries, where mainly soybean, chickpea and common
89 bean are fermented prior to consumption, alone or in association with cereals, to produce legume-
90 based fermented foods (Humblot & Guyot, 2008). Among them, Idli a traditional product from
91 India and Srilanka (Durgadevi & Shetty, 2014) is obtained by spontaneous fermentation of cereal-
92 legume mixture in which a large variety of lactic acid bacteria species such as *Leuconostoc*,
93 *Lactobacillus*, and *Streptococcus*, *Weissella*, *Pediococcus* and *Lactococcus*, but also *Bacillus* spp.
94 and yeasts were found (Mukherjee et al. 1965; Saravanan et al., 2015). Although the main actor of
95 spontaneous legume-based fermentations are mostly lactic acid bacteria and yeasts, sometimes other
96 microbial groups have been involved. For instance, *Bacillus* spp. are the main responsible of locust
97 bean and soybean spontaneous fermentation for the manufacture of traditional African and Indian
98 foods (Ouoba, et al. 2004; Sarkar, et al. 2002).
99 Recently, legumes have been used for the manufacture of novel and healthy foods as ingredient in
100 various formulations, especially combined with cereal flours. For instance, faba bean flour was used
101 to replace wheat flour in wheat-based food such as baked goods and pasta (Borsuk et al., 2012;
102 Giménez et al., 2012) or in gluten-free preparations (Han et al., 2010). The complementation
103 between cereal and legume flours is very relevant in designing novel foods since it represents the
104 easiest way to fulfil nutritional deficiencies of the cereal-based diet and to enrich the content of
105 biogenic compounds (Angioloni & Collar, 2012). Recently, lactic acid bacteria fermentation has
106 been used in combined legume-wheat flour sourdough technology providing a large biodiversity to
107 the sourdough microbiota, and a better nutritional quality of the legume-wheat bread (Rizzello et
108 al., 2015).
109 In this study, the flour obtained from two different faba bean varieties was used in traditional
110 sourdough-type biotechnology employing the backslopping procedure. According to some
111 definitions, sourdough is a mixture of flour (wheat, rye, rice, etc.) and water that is fermented with
112 lactic acid bacteria and yeasts which determine its acidifying and leavening capacity (Corsetti,
113 2013; De Vuyst & Vancanneyt, 2007; Vogel et al., 1999). Traditional sourdoughs are usually made

through multiple steps of fermentation. First, a dough, composed of flour and water, is spontaneously fermented. Subsequently, this fermented dough is used as inoculum for fermenting newly prepared dough, which, in turn, will be used as inoculum for a subsequent step of fermentation (Minervini et al., 2014), allowing the selection of a stable consortium of yeasts and lactic acid bacteria with leavening and acidifying capacity. The microbial composition of cereal mature sourdoughs from different origin has been largely investigated (Nionelli et al., 2014; Pontonio et al., 2015), while very little is known about the microbiota of sourdough-type propagation, when only legume flour is used. The structure of the flour microbiota and its metabolic activity as well as the characteristics of the flour are deeply affecting the features of the mature sourdough (Ercolini, 2013). In this perspective, the aim of this study was to investigate the microbiological and biochemical quality of faba bean flour fermentations, herein referred to as sourdough-type. The dynamics of the lactic acid bacteria community and the characteristics of the sourdough-types were monitored throughout 14 days of propagation, in order to assess their potential use in bread making.

2. Materials and Methods

2.1 Faba bean flours

Six batches of commercial Italian faba bean (Ita) (*Vicia faba major*, harvest year 2014) and six batches of Finnish faba bean (Fi) (*Vicia faba minor*, harvest year 2014) flours, obtained from the stone-milling of the dehulled seeds by CerealVeneta mills (San Martino di Lupari, PD, Italy), were pooled on the basis of the country of origin and used in this study. The proximal composition of the two flours is reported in Table 1.

2.2 Sourdough preparation and propagation

Sourdoughs were prepared and propagated through traditional protocol (sourdough type I), without use of starter cultures or baker's yeast. Flours were mixed with tap water at a ratio of 50:50 and a

final dough yield (DY) [dough weight × 100/flour weight] of 200, obtaining doughs Ita0 and Fi0 from Italian and Finnish faba bean flours, respectively. The first fermentation was carried out at 30°C for 16 h (T1), obtaining the sourdoughs Ita1 and Fi1. Successively, daily backslopping (refreshments) were carried out for 14 days, mixing 25% of the previously fermented dough with flour and water (final dough yield of 200), and incubating at 30°C for 8 h. For the analyses, aliquots of sourdoughs were also taken at 2 (Ita2/Fi2), 5 (Ita5/Fi5), 7 (Ita7/Fi7), and 14 (Ita14/Fi14) days of propagation (T2, T5, T7, and T14).

2.3 Chemical characterization

The pH value of doughs and sourdoughs was determined by a pHmeter (Model 507, Crison, Milan, Italy) with a food penetration probe. Total titratable acidity (TTA) was determined after homogenization of 10 g of dough with 90 ml of distilled water, and expressed as the amount (ml) of 0.1 M NaOH required to neutralize the solution, using phenolphthalein as indicator (official AACC method 02-31.01).

Water/salt-soluble extracts (WSE) of doughs and sourdoughs were prepared according to Weiss et al. (1993) and used to analyze organic acids, ethanol, peptides, and free amino acids (FAA). Organic acids were determined by High Performance Liquid Chromatography (HPLC), using an ÄKTA Purifier system (GE Healthcare, Buckinghmshire, UK) equipped with an Aminex HPX-87H column (ion exclusion, Biorad, Richmond, CA), and an UV detector operating at 210 nm. Elution was at 60°C, with a flow rate of 0.6 ml/min, using H_2SO_4 10 mM as mobile phase (Coda et al., 2011). The fermentation quotient (FQ) was determined as the molar ratio between lactic and acetic acids. FAA were analyzed by a Biochrom 30 series Amino Acid Analyzer as described above.

2.4 Oligosaccharides

Oligosaccharides were extracted as described by Oboh et al. (2000) with some modifications. One g of each fava bean dough (DY 200) was homogenized in 80% ethanol for 1 min at 24°C. The

166 mixture was centrifuged for 5 min at 500 g. The supernatant was decanted and the procedure
167 repeated twice on the pellet. The supernatant was freeze-dried and resuspended in 1 mL of
168 acetonitrile (65%). Each sample was analyzed using an Spherisorb-5-NH2 column (4.6 x 250,
169 Waters, USA) and an ÄKTA purifier HPLC (GE Healthcare) equipped with a refractive index
170 detector (RI-101, Perkin Elmer, USA). A solution of acetonitrile/water (65:35 v/v) was used as
171 mobile phase (flow, 1 mL/min). The identification of the sugars and the calibration curves were
172 obtained using commercial standards of sucrose, raffinose, stachyose and verbascose (Sigma
173 Aldrich, USA).
174
175 **2.5 Total phenols and antioxidant activity**
176 The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity was determined on the
177 methanolic extract (ME) of faba bean doughs. Five grams of each sample were mixed with 50 ml of
178 80% methanol to get ME. The mixture was purged with nitrogen stream for 30 min, under stirring
179 condition, and centrifuged at 4,600 × *g* for 20 min. ME were transferred into test tubes, purged with
180 nitrogen stream and stored at ca. 4°C before analysis. The concentration of total phenols was
181 determined as described by Slinkard and Singleton (1997), and expressed as gallic acid equivalent.
182 The free radical scavenging capacity was determined using the stable radical DPPH˙ (Rizzello et al.,
183 2010). The scavenging activity was expressed as follows: DPPH scavenging activity (%) = [(blank
184 absorbance – sample absorbance) / blank absorbance] x 100. The value of absorbance was
185 compared with 75 ppm butylated hydroxytoluene (BHT), which was used as the antioxidant
186 reference.
187
188 **2.6 Condensed tannins**
189 Condensed tannins were determined using the vanillin assay, as described by Price et al. (1978)
190 Samples were extracted with HCl:methanol (1:100) for 2.5 h at room temperature and centrifuged at
191 4,000 rpm for 20 min. Extracts were covered from light and analysed promptly at 30°C. Vanillin

192 reagent (equal volumes of 1% vanillin in methanol and 8% concentrated hydrochloric acid in
193 methanol) was added to extracts. Blanks were prepared by adding 4% concentrated hydrochloric
194 acid in methanol to extracts. The calibration curve was obtained using catechin and the results were
195 expressed as catechin equivalents.
196
197 **2.7 Extraction of total bacterial genomic RNA**
198 Because the diversity of metabolically active microbiota has relevant repercussions on food
199 ecosystems (e.g., fate of starter or adjunct cultures versus microbial contaminants), high throughput
200 sequencing from RNA data was adopted as it may provide a more complete description of the
201 microbiota (Ercolini, 2013). Ninety milliliters of potassium phosphate (50 mM; pH 7.0) buffer was
202 added to 10 g of sample and homogenized for 5 min, then total RNA was extracted using the
203 RiboPureTM—Bacteria Kit (Ambion RNA, Life Technologies Co., Carlsbad, CA, USA), according
204 to the manufacturer's instructions. Quality control of RNA was checked through agarose gel
205 electrophoresis. The RNA concentration was measured in a NanoDrop ND-1000 spectrophotometer
206 (NanoDrop Technologies, Rockland, DE). In order to remove DNA, the purified RNA (100 ng)
207 (final volume, 20 µl) was incubated at 42°C for 2 min in 2 µl of gDNA Wipeout Buffer 7X
208 (QuantiTect Reverse Transcription Kit, Qiagen srl, Milan, Italy) and RNase-free water (final
209 volume, 14 µl). The cDNA was obtained by the QuantiTect Reverse Transcription Kit (Qiagen)
210 according to the manufacturer's instructions. All reactions were set up in a Rotor Gene 6000
211 instrument (Corbett Life Science, New South Wales, Australia) equipped with a 36-well reaction
212 rotor.
213
214 **2.8 Pyrosequencing and data analyses**
215 Three cDNA samples, corresponding to the three batches for each dough or sourdough, were pooled
216 and used for 16S and internal transcribed spacer (ITS) based bacterial and fungal diversity analysis,
217 respectively. Microbial diversity was assessed via pyrosequencing on a Illumina MiSeq (Illumina,

Inc. San Diego, California) 2x300 flow cell at 10pM and was performed by Research and Testing Laboratories (Research and Testing Laboratories, Lubbock, TX), according to standard laboratory procedures using a two-step process. Primers targeting the V1–V3 region (*Escherichia coli* position 27–388, forward 28F: GAGTTTGATCNTGGCTCAG and reverse 388R: TGCTGCCTCCCGTAGGAGT) of the 16S rRNA gene (Francés et al., 2004; Reeder & Knight, 2010) were used for bacteria, while primers (forward ITS3F: GCATCGATGAAGAACGCAGC and reverse ITS4R: TCCTCCGCTTATTGATATGC) targeting the ITS region of fungal rRNA were used for fungi. Pyrosequencing procedures were carried out based upon RTL protocols http://www.researchandtesting.com (Lubbock, TX).

2.9 Bioinformatics

Sequenced reads for each sample were processed through denoising and chimera detection by using Research and Testing Laboratory's in-house pipeline, described at http://www.researchandtesting.com/docs/Archive/Data_Analysis_Methodology-2.2.3.pd. Briefly, sequences were grouped using their barcodes and any sequence that contained a low quality barcode or that failed to be at least half the expected amplicon length (or 250 bp, whichever was shortest) was removed from the data pool. Sequences that passed the quality filter were denoised using an algorithm based on USEARCH pipeline (Edgar, 2010), (prefix dereplication) into clusters (4% dissimilarity among sequences of the same cluster), so that each sequence of shorter length to the centroid sequence must be a 100% match to the centroid sequence for the length of the sequence. Following denoising sequences were checked for chimeras using UCHIME (Edgar et al., 2011). Finally, sequence data were separated into operational taxonomic units (OTUs) at 97% similarity using a USEARCH and all OTUs were used for classification by using UBLAST global alignment against a custom16S database comprised of well characterized sequences from nr/nt. Each sequence was corrected base by base in order to remove noise. The output was then analyzed using an internally developed Python pipeline that parses the assigned taxonomic information to create the

244 final analysis files. Alpha- and beta-diversities were evaluated by QIIME, as recently described (De
245 Filippis et al., 2013).
246 An OTU network was generated by QIIME and a bipartite graph was constructed in which each
247 node represented either a sourdough sample or a bacterial OTU. Connections were drawn between
248 samples and OTUs, with edge weights defined as the number of sequences from each OTU that
249 occurred in each sample. Networks were visualized using Cytoscape 2.5.2 (Shannon et al., 2003).
250
251 **2.10 Nucleotide sequence accession number**
252 The 16S rRNA gene sequences are available in the Sequence Read Archive of NCBI (accession
253 number BioProject 322649).
254
255 **2.11 Microbiological analyses and isolation of lactic acid bacteria**
256 Ten grams of sample were suspended in 90 ml of sterile sodium chloride (0.9%, w/v) solution and
257 homogenized with (Colworth Stomacher 400). Lactic acid bacteria were counted on MRS agar
258 (Oxoid Ltd, Basingstoke, Hampshire, UK), supplemented with 0.01 % of cycloheximide (Sigma
259 Chemical Co., USA) at 30°C for 48 h, under anaerobiosis. Yeasts were cultivated on Malt Agar
260 (Oxoid) and YM (3 g/L yeast extract, 3 g/l malt extract, 3 g/l peptone, 10 g/l dextrose)
261 supplemented with 0.01%chloramphenicol at 25°C for 48 h. Total aerophilic bacteria were
262 enumerated on PCA (Oxoid) under aerobic conditions at 30°C for 48 h and *Enterobacteriaceae*
263 were cultivated on VRBGA (Oxoid) at 37°C for 48 h.
264 Ten-fifteen colonies of presumptive lactic acid bacteria, possibly with different morphology, were
265 randomly taken from MRS plates of the highest dilutions and transferred to MRS broth. Gram-
266 positive, catalase-negative, non-motile isolates were cultivated in MRS at 30°C for 24 h, and re-
267 streaked at least twice into the agar medium. A total of 146 isolates were obtained after subculturing
268 from all propagation times.
269

2.12 Genotypic characterization and identification of lactic acid bacteria

Genomic DNA was extracted using a DNeasy® Blood and Tissue Kit (Qiagen, Germany) by following the manufacturer's instructions, with the addition of lysozyme (80 mg/ml, Sigma Aldrich, Canada) to lysis buffer solution. The obtained pure genomic DNA of isolates was stored at -20°C for RAPD and 16S rDNA sequencing analyses.

Three oligonucleotides, P1 5′- ACGCGCCCT-3′, P4 5′-CCGCAGCGTT-3′, and M13 5′-GAGGGTGGCGGTTCT-3′, (Integrated DNA Technologies, Inc. USA), with arbitrarily chosen sequences, were used for bio-typing of lactic acid bacteria isolates. Reaction mixture and PCR conditions for primers were as described by Coda et al. (2006).

Molecular weight of the amplified DNA fragments was estimated by comparison with a 1 Kb Plus DNA Ladder (Invitrogen) ranging from 100 to 12,000 bp. For random amplified polymorphic DNA (RAPD) markers, the presence or absence of fragments was recorded as 1 or 0, respectively. Only reproducible well-marked amplified fragments were scored, with faint bands being ignored. Two series of RAPD-PCR profiles were combined to obtain a unique dendrogram. Dice coefficients of similarity and UPGMA algorithm were used to estimate the similarity of the electrophoretic profiles.

To identify presumptive lactic acid bacteria, the primer pairs LacbF/LacbR was used to amplify 16S rRNA gene fragment of lactic acid bacteria (De Angelis et al., 2006). Electrophoresis was carried out on agarose gel at 1.5% (wt/vol) (Gellyphor, EuroClone) and amplicons were purified with GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare). The identification queries were fulfilled by a BLAST search in GenBank (http://www.ncbi.nlm.nih.gov/genbank/).

2.13 Statistical analysis

Sourdough propagation was carried out in triplicate and each analysis was repeated twice. Data were subjected to one-way ANOVA; pair-comparison of treatment means was obtained by Tukey's procedure at $P<0.05$, using the statistical software Statistica 8.0 (StatSoft Inc., Tulsa, USA).

296 Weighted and unweighted UniFrac distance matrices and OTU tables were used to perform
297 ADONIS and ANOSIM statistical tests through the compare_category.py script of qiime to verify
298 the microbial populations in the different samples.
299
300 # 3. Results
301 ## 3.1 Sourdough fermentation
302 Before fermentation (T0), the cell density of presumptive lactic acid bacteria in Ita and Fi doughs
303 was 3.5 ± 0.0 and 3.2 ± 0.1 log CFU/g, respectively (Fig. 1). After 16 h of fermentation (T1), cell
304 numbers of both sourdoughs significantly ($P<0.05$) increased to ca. 8.8 ± 0.0 log CFU/g. At T2, the
305 values were ca. 9.7 ± 0.1 and 9.9 ± 0.1 log CFU/g for Ita and Fi sourdoughs, respectively. From day
306 2 onward, the cell density was almost constant. Before fermentation (T0), both faba bean doughs
307 contained a low cell density of yeasts (≤ 2.0 log CFU/g), which significantly ($P<0.05$) increased
308 after the first 16 h of fermentation (ca. 3.7 ± 0.3 log CFU/g for Fi sourdough). No variation was
309 found for Ita sourdough during the first step of fermentation. After the first propagation, yeasts were
310 not anymore found in 1 g of both the sourdoughs. These results were further confirmed through
311 pyrosequencing analysis. Within first 16 h of fermentation, the cell density of *Enterobacteriaceae*
312 increased significantly ($P<0.05$) for both the sourdoughs (from ≤ 2.0 to ca. 7.4 ± 0.9 log CFU/g and
313 6.9 ± 0.4 log CFU/g for Fi and Ita sourdoughs, respectively). *Enterobacteriaceae* were not anymore
314 found in 1 g of sourdoughs from T1 onward.
315
316 ## 3.2 Biochemical characterization
317 The biochemical characteristics of faba bean doughs and sourdoughs (pH, TTA, organic acids,
318 ethanol, FQ, peptides, and TFAA) are reported in Table 2. Before the first fermentation, the pH
319 values of Ita0 and Fi0 doughs were 6.24 ± 0.12 and 6.38 ± 0.14, respectively. The pH value, which
320 significantly ($P<0.05$) decreased from the first propagation, stabilized from the T5 onward (Table
321 2). After 14 days of refreshment, Ita and Fi mature sourdoughs had pH values of 4.81 ± 0.08 to 4.90

322 ± 0.09, respectively (Table 2). Starting from T2 and for both the sourdoughs, TTA was at least
323 twice that found at T0. Low concentrations of lactic acid were detectable in T0 and progressive
324 increases were found until the seventh or the fifth day of propagation, respectively, for Ita and Fi
325 sourdoughs. Compared to Ita sourdough, the concentration of lactic acid of Fi sourdough was
326 almost 10% higher.
327 Acetic acid was not detectable before the first fermentation (Table 2). Significant ($P<0.05$)
328 increases of the acetic acid concentration were observed until T14. Overall, the highest
329 concentrations were found during propagation of Fi sourdough. Similar trends for the decrease of
330 FQ were found in both the sourdoughs. FQ remained constant from the second to the seventh day of
331 propagation, and finally decreased at T14. Ethanol was not detected at T0, and its concentration
332 significantly ($P<0.05$) increased in T1. After (from T2 to T14), ethanol concentration was
333 significantly ($P<0.05$) lower than the values found in T1 (Table 2).
334 Small fluctuations of the peptide concentration were found during propagation of Ita sourdough
335 during propagation. A progressive and significant decrease was observed for Fi sourdough. Peptides
336 in Fi14 were ca. 38% lower than Fi1 (Table 2). TFFA progressively increased during propagation.
337 Compared to corresponding doughs at T0, TFAA concentration was 58 and 106% higher in Ita5 and
338 Fi5, respectively, and 90 and 110% higher in Ita14 and Fi14, respectively.
339 Sucrose was the most abundant oligosaccharide in faba doughs. Indeed, Ita0 and Fi0 contained
340 more than 10 g/kg of sucrose (Table 3). After the first incubation, a significant ($P<0.05$) decrease
341 was found, particularly for Fi faba bean flour. Sucrose concentration gradually decreased during
342 propagation reaching a value lower than ca. 5 g/kg for both the sourdoughs after T5 (Table 3).
343 Stachyose, a tetrasaccharide consisting of two α-D-galactose units, one α-D-glucose unit, and one β-
344 D-fructose unit, was found at a concentration ranging from 2.82 ± 0.3 to 2.31 ± 0.25 g/kg in doughs
345 at T0. Its concentration progressively decreased from the first day of propagation. At the fourteenth
346 refreshment, stachyose was 75 and 56% lower than T0 respectively in Ita and Fi sourdoughs (Table
347 3). A similar trend was observed for the pentasaccharide verbascose (Table 3). Conversely, a

348 different trend was observed for raffinose. Compared to T0, its concentration was significantly
349 (P<0.05) higher in sourdough during propagation, reaching values 69 and 90% higher in Ita7 and
350 Fi5, respectively (Table 3).
351 Before fermentation (T0), Ita dough contained 0.64 ± 0.20 mmol/kg of total phenols, while Fi
352 dough was characterized by a 66% higher concentration (Table 4). Regardless of the initial content,
353 significant (P<0.05) increases were found in both the sourdoughs after the second day of
354 propagation (Table 4), reaching the highest values at T7, corresponding to an increase of ca. 30% of
355 the initial value (Table 4). Similarly, the antioxidant activity, as determined by the radical
356 scavenging activity on DPPH radical, progressively increased from the first to the fifth day of
357 propagation, remaining stable at values higher than 82.9 ± 1.5 and 85.2 ± 1.0 % respectively for Ita
358 and Fi sourdoughs.
359 A marked difference in condensed tannins was found between the two sourdoughs. At T0, Fi dough
360 contained 328.29 ± 12 mg/100g of tannins (expressed as catechin equivalents), while a value 10
361 times lower was found for Ita dough (Table 4). Significant (P<0.05) decreases were found from the
362 second day of propagation, and the concentration became stable after seven (It) or five (Fi) days of
363 propagation (Table 4). At T14, condensed tannins were ca. 50 and 30% lower than T0, respectively
364 for Ita and Fi sourdoughs.
365
366 **3.3 Pyrosequencing data analysis and alpha diversity**
367 A total of 171,818 and 133,554 quality-trimmed sequences of 16S rRNA gene amplicons were
368 obtained from Ita (average length 365 bp) and Fi (average length 363 bp) doughs or sourdoughs,
369 respectively. The number of OTU, the Chao1 and Shannon indices, and the richness estimator
370 (ACE) are reported in Table 1S in the supplemental material. Good's estimated sample coverage
371 (median value of ca. 98%; P<0.05) and the rarefaction curves (see Figure 1S in the supplemental
372 material) indicated that a satisfactory coverage was reached for all the samples analyzed.

373 Among Ita samples, the lowest microbial diversity was found for dough Ita0. Suddenly after the
374 first fermentation (Ita1), the diversity became the highest. From T2, the diversity indices decreased
375 and remained almost constant throughout propagation. The trend was almost similar for Fi samples,
376 except for the highest diversity which was found after two days of propagation (Fi2) and then
377 simplified through propagation. Overall, microbial diversity was markedly simplified after 5 days of
378 propagation and it remained almost constant at 14 days.
379 Metabolically active bacteria were also analyzed using three phylogeny-based beta-diversity
380 measures (Figure 2). The principal coordinate analysis (PCoA) based on the unweighted UniFrac
381 distance matrix clearly differentiated the two doughs (Ita0 and Fi0) based on the geographic origin
382 of the flour. After the first 16 h of fermentation, sourdoughs were distributed on the opposite part of
383 the plane. Ita1 and Fi1 sourdoughs perfectly overlapped and, with the only exception of Fi2, all the
384 other Italian and Finnish sourdoughs grouped together.
385
386 **3.4 Structure and changes of the microbiota during sourdough propagation**
387 The bacterial sequences from RNA assigned to bacterial phyla and their relative abundances (%)
388 varied slightly depending on geographical origin of the flour, and number of propagations (Figure
389 2S). *Proteobacteria* was the only phylum found in both Italian and Finnish doughs, prior the first
390 fermentation. Although with different relative abundances (%), soon after the first fermentation,
391 *Proteobacteria* were flanked by *Firmicutes* in both Ita (50.6%) and Fi (14.5%) sourdoughs. After
392 two days of propagation, Ita2 was completely dominated by *Firmicutes*, whereas *Proteobacteria*
393 were still persistent (18.8%) in Fi2. As shown by RNA analysis, only *Firmicutes* dominated after 5,
394 7 and 14 days of propagation of both Ita and Fi sourdoughs. According to alpha- and beta-diversity,
395 and considering the 30 most dominant genera of all samples, Ita and Fi doughs and sourdoughs
396 were distributed in four clusters (Figure 3). Clusters I and II encompassed Ita0 and Fi0 doughs,
397 respectively. Cluster III grouped both sourdoughs after the first fermentation (Ita1 and Fi1). With
398 the only exception of sourdough Fi2 (cluster IV, sub-cluster A), all sourdoughs from T2 to T14

399 clustered together. Ita0 was completely dominated by *Sphingomonadaceae*, which were flanked by
400 very low abundances of *Enterobacteriales*, *Enterobacteriaceae* and *Pseudomonas* in Fi0. After the
401 first fermentation (Ita1), the bacterial profile changed and became dominated by *Sphingomonas*
402 (36.7%), *Pediococcus* (16.1%), *Lactobacillales* (12.2%), *Escherichia* (8.2%) and *Weissella* (1.2%).
403 Two days of propagation were needed to markedly change the bacterial profile of sourdough Ita2,
404 which was dominated by *Pediococcus* (42.9%), *Leuconostoc* (32.8%) and *Weissella* (24.1%). This
405 dominance remained almost constant during propagation. A higher diversity was found in Finnish
406 faba bean sourdough compared to the Italian ones. *Weissella* (26.9%), *Escherichia* (16.7%),
407 *Enterobacteriales* (15.9%), *Leuconostoc* (11.9%), *Pediococcus* (11.8%), *Lactobacillales* (7.6%),
408 *Lactococcus* (5.2%) and *Enterobacteriaceae* (3.8%) were found after two days of propagation.
409 From T5 onward, the bacterial diversity simplified and sourdoughs were dominated only by genera
410 (*Leuconostoc*, *Pediococcus*, *Weissella, Lactococcus* and *Lactobacillus*) belonging to *Firmicutes*
411 phylum. *Lactococcus Leuconostoc* and *Lactobacillus* were found at the highest incidence. After 14
412 days of propagation, *Leuconostoc* (58.3%) still dominated, and *Lactobacillus* was still detected
413 (13.2%), even in the presence of *Pediococcus* (28.5%). Taxonomic details up to the species level
414 were supplied where such assignment was possible (data not shown). For both Ita and Fi
415 sourdoughs, the taxonomic assignment up to species level within the *Firmicutes* phylum was
416 possible only for *Weissella cibaria* and *Pediococccus pentosaceus*. Starting from Ita1 to Ita14, the
417 incidence of these two species varied from 1.2 and 16.2% (Ita1) to 37.4% and 7.0% (Ita14),
418 respectively. Regarding Fi sourdoughs, *W. cibaria* appeared after the first fermentation (7.9%) and
419 disappeared at T7 (22.8%), whereas *P. pentosaceus* was found from T2 (11.8%) onward (28.4%).
420 A total of 146 presumptive lactic acid bacteria were isolated from MRS agar at the highest dilution
421 plates and subjected to RAPD-PCR analysis and 16SrRNA sequencing (Figure 4A and B). Isolates
422 identified through culture-dependent methods almost reflected the lactic acid bacteria microbiota
423 retrieved by RNA pyrosequencing. Particularly, isolates from Ita sourdough belonged to *P.*
424 *pentosaceus* (43 isolates, 57.3%), *Leuconostoc mesenteroides* subsp. *mesenteroides* (23 isolates,

425 30.7%), and *Weissella koreensis* (9 isolates, 12%). Lactobacilli were not identified. Lactic acid
426 bacteria from Fi sourdough mostly belonged to *P. pentosaceus* (32 isolates, 45.1%), *Leuconostoc*
427 spp. (17 isolates 24% of which 15 isolates were *L. mesenteroides* subsp. *mesenteroides*), *W.*
428 *koreensis* (6 isolates, 8.5%), *Lactobacillus sakei* (5 isolates, 7%), *Enterococcus* spp. (8 isolates,
429 11.2%), *W. cibaria* (ca. 2 isolates, 2.8%), and *Lactococcus lactis* subsp. *lactis* (1 isolate, 1.4%),
430 All the isolates were grouped together at a similarity level of ca. 54% and 51% for Ita and Fi
431 sourdoughs, respectively. At the similarity level of 80%, the isolates from both the sourdoughs were
432 clustered in eight groups (A-H) except for I01, F142, F145, F19, F08, F110, F06, and F09, which
433 were not grouped.
434
435 **4. Discussion**
436 Nowadays, legume flours are employed for an increased number of novel food applications,
437 including sourdough biotechnology and baking, aiming at fully exploit the potential of these
438 nutritious crops (Multari et al., 2015; Rizzello et al., 2015). In this perspective the ecological
439 dynamic of legume flour fermentation can provide useful information for baked goods production.
440 In this study, the microbial community and biochemical properties of two varieties of faba bean
441 sourdough-type fermentations were evaluated during backslopping procedure. The flours used were
442 obtained from two faba bean cultivars grown for food and feed uses: *V. faba major* (named "broad
443 bean") including cultivars with large flattened seeds, popular in the southern regions of Europe, and
444 *V. faba minor* (named "field bean" or "horse bean"), including cultivars with medium to relatively
445 small and round seeds (Crépon et al., 2010).
446 As shown by PCoA, which was based on the unweighted UniFrac distance matrix of the number of
447 OTUs, Ita and Fi flours and doughs, before fermentation, were contaminated by metabolically
448 active bacteria, most likely representing the outcome of milling procedure.
449 The initial community of the two flours, before fermentation, was dominated by a metabolically
450 active phylum, which likely represented the outcome of environmental contamination. Usually,

451 Proteobacteria are found in wastewater, forage feed, and soils (Benedek et al., 2013). Members of
452 the genus *Sphingomonas* and their closely related species constitute a significant fraction of the
453 phyllosphere population of healthy plants making them the core phyllosphere community that
454 protect plants against pathogens (Innerebner et al., 2011). *Sphingomonas* strains are associated with
455 *Leguminosae* (Rivas et al., 2004).

456 The microbiota of Ita and Fi doughs before the second day of propagation mirrored the differences
457 between the two flours mostly due to small hulls fragments contained in the flour from Finnish
458 origin as a consequence of the milling procedure. Indeed, due to the smaller size and the peculiar
459 shape of the *V. faba minor* (Finnish) seeds, the mechanical dehulling process, which lead to the
460 removal of the external layer of the seed, was less efficient compared to the Italian *V. fava major*.
461 Whereas *Sphingomonadaceae* was the only family harbored in the Ita dough, *Enterobacteriaceae*
462 and *Pseudomonas* spp. were found in Fi dough. Soon after the first fermentation this population was
463 almost completely inhibited. The only exception found in Ita and Fi sourdoughs were represented
464 by the *Sphingomonas* and *Enterobacteriaceae* family, respectively. The latter contaminant even
465 increased during early propagations and was found in Fi sourdoughs until 2 days.
466 *Enterobacteriaceae* grew, probably survived because of a certain tolerance to acid stress. Similarly,
467 *Enterobacteriaceae* contaminated and persisted during durum wheat sourdough propagation
468 (Ercolini, 2013). Besides the influence on microbiota, the presence of hulls, characterized by high
469 concentration of tannins and dietary fibers (Vilariño et al., 2009), impacted also on other
470 biochemical properties of Fi doughs. Overall, higher microbial diversity was found in Fi than in Ita
471 sourdough, probably due to the higher microbial contamination related to the hulls surface, even
472 though the diversity markedly decreased with increasing propagation steps.

473 The propagation conditions of faba bean sourdoughs chosen in this study were similar to traditional
474 protocols previously used for cereal and cereal-legumes sourdough fermentation (Ercolini, 2013;
475 Minervini et al., 2012; Rizzello et al., 2014). After the first 16 h of fermentation, lactic acid bacteria
476 dominated the sourdough reaching a cell density of 9 log CFU/g, which remained almost constant

477 from the second day onward, indicating the stability of the environment, as largely observed for
478 cereal sourdoughs or cereal-legume mixtures, such as idli (De Vuyst & Neysens, 2005; Ercolini,
479 2013; Saravanan et al., 2015; Van der Meulen et al., 2007). The evolution of yeasts was simpler. As
480 shown by plate count and culture independent methods, after the second backslopping, yeasts were
481 not detected anymore. A similar trend was previously observed during other spontaneous legume
482 fermentations (Granito & Álvarez, 2006), and a very low yeast cell density was commonly found
483 after 5 and 10 days of propagation also in bean, chickpea and wheat-legume sourdoughs (Rizzello et
484 al., 2014). Overall, the spontaneous fermentation of vegetables and fruits includes the succession of
485 hetero- and homo-fermentative lactic acid bacteria, with or without yeasts (Plengvidhya et al.,
486 2004).
487 As shown by the pseudo-heat map depicting bacterial diversity at genera level, *Pediococcus,*
488 *Leuconostoc,* and *Weissella* were already the dominant genera at the second day of propagation,
489 while only a low abundance of *Lactobacillus* in Fi, previously isolated from wheat–legume
490 sourdough and typical of cereal sourdough, was observed (Corsetti & Settanni, 2007; De Vuyst &
491 Neysens, 2005 Rizzello et al., 2014). Subsequently, these genera stably dominated both sourdoughs
492 during propagation. A similar scenario was already found during fermentation of different plant
493 matrices, including fermented beans, in which pediococci can multiply rapidly and become a major
494 component of the lactic acid bacterial population in association with members of *Lactobacillus*,
495 *Leuconostoc* and *Weissella* genera (Holzapfel et al., 2006). The stable persistence of lactic acid
496 bacteria genera in cereal-based sourdough was attributed to environmental adaptation (Ercolini et
497 al., 2013) and, especially, to the synthesis of antimicrobial compounds (Nam et al., 2012).
498 Among the isolates, *Pediococcus* and *Leuconostoc* spp. had the highest frequency of occurrence in
499 both sourdoughs. Compared to Ita sourdough, in which only *W. koreensis* was retrieved together
500 with *P. pentosaceus* and *Leuconostoc* spp, the presence of *Enterococcus* spp., *Lb. sakei*, *W. cibaria*,
501 *and Lc. lactis* was also detected in Fi sourdough. Almost all the species isolated were previously
502 identified in cereal sourdoughs (De Vuyst & Neysens, 2005; De Vuyst et al., 2014; Minervini et al.,

503 2012), with the exception of *W. koreensis*, which was isolated mainly from kimchi, a traditional
504 Korean fermented-vegetable food (Lee et al., 2002; Moon et al., 2012). Enterococci have ubiquitous
505 nature, and their higher occurrence in Fi doughs can be due to the presence of the hulls, as a
506 consequence of farming practices and contamination with animal faeces (eg. manure) (Franz et al.,
507 1999; Giraffa, 2003). However, enterococci were not anymore present after the second day of
508 propagation. Similar results were previously reported studying the community dynamics of bacteria
509 in wheat sourdough fermentation (Weckx et al., 2010), where *Enterococcus* spp., found during a
510 transition phase of propagation, disappeared since not able to survive to a long-term acidification
511 process (Weckx et al., 2010).
512 *Lb. sakei* was the only lactobacillus isolated from the first to the last day of propagation. This lactic
513 acid bacteria can be retrieved from several fermented food including cereal sourdoughs (Lee et al.,
514 2005; Scheirlinck et al., 2007), and it is commonly found in kimchi in association with other
515 lactobacilli, leuconostocs and weissellas (Kim & Chun, 2005). The genus *Leuconostoc* has been
516 found to predominate on many plant materials together with lactobacilli and, occasionally,
517 *Weissella* spp. (Björkroth & Holzapfel; 2006; Mundt et al., 1967). *Leuc. mesenteroides* subsp.
518 *mesenteroides* is often isolated from vegetables such as beans and peas for freezing (Sharpe &
519 Pettipher, 1983). It is worth noticing that *P. pentosaceus* and *Leuc. mesenteroides* commonly
520 constitute the microbiota involved during the first stage of kimchi fermentation (as reviewed by Di
521 Cagno et al., 2013). Overall, the high abundance of *P. pentosaceus* and *Leuc. mesenteroides* isolates
522 in both faba bean sourdoughs might be a result of relatively high pH values, confirming the
523 influence of flour on the establishing ecosystem (Minervini et al., 2014).
524 The network-based analyses were used to map sourdough microbial community composition (RNA
525 data) onto time of propagation and type of flour (Figure 5). It provided a novel and immediate
526 interpretation of the dynamics during sourdough preparation. Overall, regardless the type of flour
527 used (Finnish or Italian), doughs prior the fermentation (red color) and those soon after the first
528 fermentation (green color) were characterized by the highest microbial diversity. The microbial

529 complexity simplified through the propagation as suggested by the reduced number of OTU
530 characterizing each dough or sourdough. Similar trend was already reported for cereal-based
531 sourdoughs (Ercolini et al., 2013). Moreover, the shared OTU, meaning those facing towards the
532 inside of the network and connected to others, increased during the propagation, highlighting that
533 sourdoughs became more closely associated with one another, based on presence and abundance of
534 dominant lactic acid bacteria. OTU network clearly distinguished different types of flours and
535 sourdoughs at different stages of propagation according to the complexity of the microbiota. The
536 core microbiota, shared between sourdoughs at the end of fermentation, appeared clearly
537 differentiated (Figure 5).
538 The presence of hulls and the difference in the microbiota composition of the two flours were
539 reflected also in the acidification of the sourdoughs, and markedly at the beginning of propagation.
540 Organic acids, particularly acetic acid content, were almost constantly higher in Fi sourdough, and,
541 consequently FQ was lower throughout the propagation time. A possible reason is the higher fiber
542 content and the different carbohydrate profile of Fi compared to Ita flour, including, for example,
543 different amount of RFOs. It has been previously discussed that, the question whether RFOs are
544 anti-nutrititional factors or functional ingredients stimulating growth of beneficial intestinal bacteria
545 dependson their dose (Oku & Nakamura, 2002; Van Loo et al., 1999). It has been estimated that
546 intake of 0.3 g/kg body weight per day of non-digestible oligosaccharides is tolerated without the
547 adverse side effects deriving from legumes consumption (Oku & Nakamura, 2002). As a
548 consequence, the reduction of oligosaccharide content may lead to a health benefit due to the
549 transformation of RFOs into “functional ingredients” (Teixeira et al., 2012). Many lactic acid
550 bacteria, including *Lactobacillus* and *Leuconostoc* spp, produce α-galactosidase (α-Gal) and are
551 able to eliminate RFOs in food prepared from soy, beans, cowpea, pea flours (Coda et al., 2015;
552 Curiel et al., 2015; Teixeira et al., 2012). During faba bean flour sourdough propagation, a marked
553 decrease of the RFOs stachyose and verbascose was found, especially in Ita sourdoughs.
554 Nevertheless, a slight increase in raffinose concentration was found in the intermediate days of

propagation, for both the flours, probably released from the partial hydrolysis of verbascose and stachyose (Teixeira et al., 2012) and not further utilized. In fact, pediococci, particularly abundant in Ita sourdough, cannot ferment raffinose (Huys et al., 2011), thus contributing to its accumulation. Generally, despite the original different chemical composition of the two flours, the biochemical development of the sourdoughs followed similar trends. During sourdough propagation the peptide content decreased and free amino acid concentration increased in both the flours, even though some differences were found. However, while the final peptide concentration had similar value in both the sourdoughs, the total free amino acid amount was higher in Fi throughout propagation time, thus hypothesizing a different contribution of the endogenous proteolytic enzymes and/or a different proteolytic activity of the dominant lactic acid bacteria strains. In particular, several studies showed that *L. sakei* strains (only found in Fi sourdoughs) possess an efficient proteolytic system consisting in a transport system for oligopeptides (Opp), as well as a di/tripeptides ABC transport system including five subunits (DppA/P, DppB, DppC, DppD and DppF) and a di/tripeptides ion-linked transport system (DtpT), and 18 peptidases with different specificities (unique aminopeptidases, endopeptidases, di/tripeptidases and proline peptidases) (Sinz & Schwab, 2012).

In both the sourdoughs, an increase of total phenols and antioxidant activity was found at the end of propagation time. As previously shown (Nionelli et al., 2014; Rizzello et al., 2013; 2016), lactic acidification improves the extraction of total phenols. Esterase activities, able to hydrolyze complex phenolic compounds and their glycosylated forms into the corresponding phenolic acids during sourdough fermentation were largely described for lactic acid bacteria (Esteban-Torres et al., 2013; Nionelli et al., 2014). The increased solubilization of phenolics might be related to the highest antioxidant activity found in sourdoughs. At the same time, condensed tannins concentration decreased of 30-50% in Fi and Ita sourdoughs, after 14 days. Condensed tannins, the most abundant form of tannins in faba bean, are mostly concentrated in the hulls. They are composed of flavonoid units and responsible for the decrease of the protein digestibility and the formation of protein-tannin complexes, (Kosińska et al., 2011). As previously observed, during fermentation with lactic acid

bacteria, degradation products of tannins can contribute to the increase of total phenols amount (Coda et al., 2015).

The evolution of the microbiota during the propagation of sourdough made with faba bean flour was investigated for the first time in this study. A strong similarity with plant based fermentation, particularly with kimchi, was observed in the type and association of microorganisms. Compared to the microbial dynamics previously reported for cereal sourdough, the absence of key microorganisms like *Lactobacillus plantarum* and *Saccharomyces cerevisiae* at the advanced steps of propagation emerged as the main difference However, it is not possible to define the exact role of the factors leading to the dominance of certain lactic acid bacteria species upon faba bean sourdough backslopping in comparison with cereal-based matrices.A combination of several factors must be considered for the survival, succession, and dominance of lactic acid bacteria species in spontaneous sourdough propagation (Di Cagno et al., 2014; Lee et al., 2002; Minervini et al., 2014; Vogelmann & Hertel, 2011). Some of these factors, like the pH range and buffering capacity, the enzymatic activity, the fermentable carbohydrate profile, the high concentration in condensed tannins and RFOs are also strongly diversifying legume and cereal matrix. Moreover, the residual hulls, unavoidably present in *V. faba minor* (Finnish) flour, strongly affected the microbial diversity and the biochemical characteristics of the mature sourdough compared to the *V. faba major* (Italian) flour.

Although further experimental approaches might clarify the mechanisms involved in lactic acid bacteria dominance in faba bean sourdough, the results here collected provide information useful for a proper selection of starters, and the application of sourdough fermentation, recognized as an emerging and promising biotechnology for improving nutritional and functional features of faba bean flour.

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831

Legends to figures

Figure 1. Cell density of LAB, yeasts, and *Enterobacteriaceae*, of Italian (Ita) and Finnish (Fi) faba bean doughs and sourdoughs (A and B, respectively) during propagation (T0, T1, T2, T5, T7 and T14). The error bars indicate the standard deviations of the results analyzed in duplicates.

Figure 2. Principal coordinate analysis (PCoA) based on weighted UniFrac analysis of all 16S RNA gene sequences of Italian and Finnish faba bean doughs (after mixing and before fermentation) (Ita0, Fi0) and sourdoughs after 1 (Ita1, Fi1), 2 (Ita2, Fi2), 5 (Ita5, Fi5), 7 (Ita7, Fi7), and 14 (Ita14, Fi14) days of propagation.

Figure 3. Heat map summarizing the relative abundances of the 30 most dominant genera in RNA samples directly extracted from Italian and Finnish faba bean doughs (after mixing and before fermentation) (Ita0, Fi0) and sourdoughs after 1 (Ita1, Fi1), 2 (Ita2, Fi2), 5 (Ita5, Fi5), 7 (Ita7, Fi7), and 14 (Ita14, Fi14) days of propagation. The colour key defines the percentages of OTUs in the samples. Bacterial genera and samples are sorted based on Euclidean distances and weighted UniFrac distances, respectively.

Figure 4. Dendrograms obtained by combined random amplification of polymorphic DNA patterns for the isolates from Italian (Ita) and Finnish (Fi) faba bean doughs and sourdoughs (A and B, respectively) during propagation (T0, T1, T2, T5, T7 and T14) using primers M13, P4 and P7. Cluster analysis was based on the simple matching coefficient and unweighted pair group with arithmetic average.

Figure 5. Simplified illustration of possible sourdough-microbe networks based on RNA data. Network diagrams are color- and symbol- coded by time of propagation and type of flour. Samples: Italian (square) and Finnish (triangle) faba bean doughs (prior to fermentation and before becoming sourdough) (Ita0 and Fi0, red colour); sourdoughs after 1 (Ita1 and Fi1, green colour), 2 (Ita2 and

856 Fi2, blue colour), 5 (Ita5 and Fi5, pink colour), 7 (Ita7 and Fi7, orange colour) and 14 (Ita14 and
857 Fi14, yellow colour) days of propagation.
858

859 **Table 1.** Proximal composition of Italian (*Vicia faba major*) and Finnish (*Vicia faba minor*, cv
860 Kontu) faba bean flours.
861

	Faba bean flours	
	Italian	**Finnish**
Dry matter (%)	87.99±0.03[b]	89.07±0.18[a]
Fat (%)	1.43±0.01[a]	1.28±0.04[b]
Protein (%) (N × 5.7)	24.11±0.19[b]	25.82±0.18[a]
Total carbohydrate (%)	58.51±0.68[a]	42.55±0.77[b]
Starch (%)	44.83±0.16[a]	38.18±0.70[b]
Dietary fiber (%)	9.90±0.36[b]	20.97±0.15[a]
Ash (%)	3.52±0.00[b]	3.65±0.02[a]

862 The data are the means of three independent experiments ± standard deviations (n=3).
863 [a-b] Values in the same row with different superscript letters differ significantly ($P < 0.05$)

Table 2. Chemical characteristics of the sourdoughs obtained from Italian (Ita) and Finnish (Fi) faba bean flours. Sourdough propagation was carried out for 14 days. T0 corresponds to doughs before fermentation; T1 to doughs after the first fermentation (30°C for 16h). After T1, refreshments were carried out daily, by mixing 25% of the previously fermented dough with flour and water, and incubating at 30°C for 8 h. T2, T5, T7, and T14 correspond to the sourdoughs analyzed at 2, 5, 7, and 14 days of propagation.

		T0	T1	T2	T5	T7	T14
pH	Ita	6.24±0.12^{a}	5.95±0.04 ^{b}	4.89±0.13^{c}	4.80±0.10^{d}	4.82±0.10^{d}	4.81±0.08^{d}
	Fi	6.38±0.14^{a}	5.74±0.11 ^{b}	5.00±0.11^{c}	4.91±0.10^{c}	4.87±0.09^{c}	4.90±0.09^{c}
TTA (mL 0.1N	Ita	5.80±0.14^{c}	6.00±0.12^{c}	15.40±0.12^{b}	15.80±0.13^{a}	16.00±0.14^{a}	16.20±0.11^{a}
NaOH /10 g)	Fi	7.40±0.13^{e}	8.60±0.11^{d}	15.00±0.15^{c}	16.20±0.14^{b}	16.40±0.14^{b}	17.00±0.13^{a}
Lactic acid (mmol/kg)	Ita	13.31±0.60^{e}	28.53±1.50^{d}	86.24±1.80^{c}	98.12±2.20^{b}	103.92±2.60^{a}	103.40±2.50^{a}
	Fi	16.79±1.05^{d}	26.98±1.60^{c}	92.05±2.80^{b}	113.55±2.90^{a}	110.5±3.70^{a}	107.55±3.80^{a}
Acetic acid (mmol/kg)	Ita	nd	2.44±0.50^{d}	12.17±1.50^{c}	14.02±1.60^{b}	15.37±1.40^{b}	17.73±1.40^{a}
	Fi	nd	5.86±0.30^{d}	14.99±1.00^{c}	17.49±2.00^{b}	19.85±2.00^{b}	24.79±2.00^{a}
FQ	Ita	-	11.67^{a}	7.09^{b}	7.00^{b}	6.76^{b}	5.83^{c}
	Fi	-	4.61^{c}	6.14^{a}	6.49^{a}	5.57^{b}	4.34^{c}
Ethanol (mmol/kg)	Ita	nd	31.11±1.0 ^{a}	23.96±1.55 ^{b}	21.19±1.60 ^{b}	20.35±1.70 ^{b}	14.20±1.60 ^{c}
	Fi	nd	32.68±1.20 ^{a}	23.89±1.50 ^{b}	18.86±2.00 ^{c}	17.75±1.80 ^{c}	14.75±1.50 ^{d}
Peptides (g/kg)	Ita	35.90±3.75^{a}	32.75±3.50^{a}	27.15±3.50^{b}	30.55±3.75^{a}	30.72±3.75^{a}	29.00±3.50^{b}
	Fi	57.92±4.25^{a}	38.42±2.50^{b}	41.75±2.75^{b}	39.45±3.10^{b}	29.92±3.37^{c}	23.70±2.50^{d}
Total free amino acids (mg/kg)	Ita	3205±48^{d}	4785±37^{c}	4744±50^{c}	5064±35^{b}	5070±40^{b}	6106±51^{a}
	Fi	3751±40^{e}	7551±33^{d}	7611±43^{c}	7740±35^{b}	7708±56^{b}	7859±50^{a}

The data are the means of three independent experiments ± standard deviations (n=3). $^{a\text{-}e}$ Values in the same row with different superscript letters differ significantly ($P < 0.05$)
nd : not detected

Table 3. Oligosaccharides concentration in sourdoughs obtained from Italian (Ita) and Finnish (Fi) faba bean flours. Sourdough propagation was carried out for 14 days. T0 corresponds to doughs before fermentation; T1 to doughs after the first fermentation (30°C for 16h). After T1, refreshments were carried out daily, by mixing 25% of the previously fermented dough with flour and water, and incubating at 30°C for 8 h. T2, T5, T7, and T14 correspond to the sourdoughs analyzed at 2, 5, 7, and 14 days of propagation.

		T0	T1	T2	T5	T7	T14
Sucrose (g/kg)	Ita	13.70±0.45^{a}	10.43±0.70^{b}	8.04±0.50^{c}	5.16±0.80^{d}	4.60±0.50^{d}	4.45±0.50^{d}
	Fi	10.99±0.80^{a}	5.65±0.44^{b}	6.03±0.45^{b}	5.10±0.50^{c}	4.78±0.35^{c}	4.67±0.40^{c}
Raffinose (g/kg)	Ita	0.83±0.015^{e}	1.02±0.020^{d}	1.29±0.025^{b}	1.26±0.040^{b}	1.40±0.045^{a}	1.11±0.040^{c}
	Fi	1.21±0.050^{b}	2.28±0.040^{a}	2.30±0.050^{a}	2.30±0.055^{a}	1.18±0.045^{b}	0.79±0.050^{c}
Stachyose (g/kg)	Ita	2.82±0.30^{a}	0.99±0.15^{b}	0.92±0.11^{b}	0.75±0.10^{c}	0.76±0.10^{c}	0.72±0.15^{c}
	Fi	2.31±0.25^{a}	1.86±0.12^{b}	1.50±0.08^{b}	1.03±0.10^{c}	1.06±0.15^{c}	1.02±0.10^{c}
Verbascose (g/kg)	Ita	2.25±0.15^{a}	0.86±0.10^{b}	0.79±0.08^{c}	0.78±0.08^{c}	0.64±0.10^{d}	0.62±0.15^{d}
	Fi	2.53±0.30^{a}	1.66±0.20^{b}	1.20±0.15^{c}	1.18±0.10^{c}	1.09±0.10^{d}	1.09±0.12^{d}

The data are the means of three independent experiments ± standard deviations (n=3). $^{a\text{-}e}$ Values in the same row with different superscript letters differ significantly ($P < 0.05$)

Table 4. Total phenols, antioxidant activity and condensed tannins in sourdoughs obtained from Italian (Ita) and Finnish (Fi) faba bean flours. Sourdough propagation was carried out for 14 days. T0 corresponds to doughs before fermentation; T1 to doughs after the first fermentation (30°C for 16h). After T1, refreshments were carried out daily, by mixing 25% of the previously fermented dough with flour and water, and incubating at 30°C for 8 h. T2, T5, T7, and T14 correspond to the sourdoughs analyzed at 2, 5, 7, and 14 days of propagation.

		T0	T1	T2	T5	T7	T14
Total phenols (mmol/kg)	Ita	0.64±0.020^{c}	0.66±0.030^{c}	0.76±0.050^{b}	0.74±0.050^{b}	0.80±0.070^{a}	0.86±0.060^{a}
	Fi	1.06±0.070^{c}	1.09±0.040^{c}	1.28±0.055^{b}	1.31±0.044^{b}	1.37±0.048^{a}	1.41±0.060^{a}
Antioxidant activity (%)	Ita	78.0±1.0^{c}	77.8±1.5^{c}	81.3±1.0^{b}	82.9±1.5^{a}	83.6±1.5^{a}	84.3±1.0^{a}
	Fi	81.6±0.8^{c}	83.6±0.8^{b}	84.6±1.0^{a}	85.2±1.0^{a}	86.4±1.2^{a}	87.4±1.0^{a}
Condensed tannins (mg/kg)	Ita	232.3±01.5^{a}	223.1±01.8^{a}	175.3±01.2^{b}	147.8±02.2^{c}	113.8±02.5^{d}	113.9±01.9^{d}
	Fi	3282.9±31.2^{a}	3017.9±40.1^{a}	2795.3±23.4^{b}	2309.0±23.2^{c}	2362.0±25.0^{c}	2305.2±15.4^{c}

The data are the means of three independent experiments ± standard deviations (n=3).
$^{a-d}$ Values in the same row with different superscript letters differ significantly ($P < 0.05$)